

Extracellular vesicles mirna-126: A potential therapeutic role in type i diabetic rat model

Extracellular vesicles/-miRNA-126/therapeutic role/diabetes

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Abstract

Aim: Type 1 diabetes mellitus (T1DM) is a metabolic disorder of an unknown etiology caused mainly by autoimmune or viral destruction of pancreatic β -cells. So far, the main therapy is insulin administration with no curable treatment. The aim of the current study was to evaluate the therapeutic effect of extracellular vesicles (EVs) derived from bone marrow mesenchymal stem cells (BM-MSCs) and transfected with miRNA126 in treatment of T1DM in rats. Moreover, to compare transfected EVs effect versus their parent BM-MSCs and untransfected EVs.

Material and Methods: The induction of diabetes in rats was done by intraperitoneal (i.p) injection of streptozotocin (STZ). Rats were divided randomly into groups that received either BM-MSCs or EVs or miRNA-126 mimic transfected EVs or miRNA-126 inhibitor transfected EVs.

Results: miRNA-126 mimic transfected EVs, BM-MSCs, EVs treated groups resulted in lowering of blood glucose level and increase in plasma insulin level significantly compared to diabetic untreated group with significantly higher miRNA126 level in pancreatic tissue of miRNA126 mimic-EVs treated group compared to diabetic group. Results were superior in miRNA-126 treated group compared to BM-MSCs treated.

Discussion: miRNA-126 transfected EVs were found to be superior to BM-MSCs in the treatment of STZ induced type-1 DM. MSCs derived EVs may represent a novel cell-free treatment strategy with a potential implication of miRNA-126.

Keywords

miRNA-126, Extracellular Vesicles, Bone Marrow Mesenchymal Stem Cells, Diabetes Mellitus

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Introduction

Type 1 DM (T1DM) is considered as one of the most common metabolic disorders in childhood. Different treatment modalities were developed for the treatment of T1DM, however due to patient inconvenience and reported their complications and patient inconvenience more studies is needed to eradicate the disease [1].

Bone marrow mesenchymal stem cells (BM-MSCs) are non-hematopoietic stem cells which can produce and secrete wide spectrum of cytokines, chemokines, and growth factors in a paracrine fashion that affect the action of nearby cells [2].

Extracellular vehicles (EVs) derived from MSCs is considered as one of the promising paracrine mechanisms of stem cells that could effectively mimic the beneficial effects of MSCs. EVs mainly contribute to the action of stem cells transferring proteins, lipids, and RNA species between cells. Many studies have assessed the potential therapeutic of MSCs derived EVs in T1DM due to their immunoregulatory actions [3].

MicroRNAs (miRNAs) are considered as a non-coding small-molecule RNAs that control gene expression by attaching to complementary target sequences of the 3' untranslated regions (3'-UTR) of mRNAs [4]. miRNAs may have cross-talks with mRNAs in pancreatic tissues of T1DM [5].

MiRNA-126 is profusely found in endothelial cells in and performs an important role in regulating vascular integrity and angiogenesis regulation. Hence, several studies have investigated its potential therapeutic role in decreasing the consequences of diabetic vascular complications in various types of DM [6]. However, no study, up to now, have studied the potential paracrine role of miR-126 on pancreatic tissue regeneration and insulin production.

The current study aimed to assess the potential therapeutic role of EVs secreted from bone marrow-derived mesenchymal stem cells (BM-MSCs) in the treatment of T1DM as compared to BM-MSCs alone. Moreover, to study miR-126 as a potential molecular mechanism by which EVs communicate with pancreatic tissue. As far as we know, this is the first study to assess the use of BM-MSCs- derived EVs in the treatment of DM with the implication of miRNA 126.

Material and Methods

Laboratory animals

The present study included sixty adult male Wistar albino rats (150-180 g, 7-8 weeks). Rats were housed under a standard condition as 14 to 10 h light-dark cycle at $25 \pm 2^\circ\text{C}$ with free access to food and water, at the institutional Animal House of Physiology Department. Experiments were done in compliance with the approved guidelines set by the institutional Research Ethics Committee, the National Institutes of Health guide for the care and use of laboratory animals.

Experimental design

Bone marrow isolation

Two male Wistar albino rats (25-35 g, 3 weeks) were sacrificed for bone marrow extraction. The femur and tibia were dissected, disinfected, and flushed with PBS. The proximal and distal ends of the tibiae and femurs were cut [7].

Preparation of bone marrow derived mesenchymal stem cells (BM-MSCs)

The cells obtained from the tibia and femur bones were layered on Ficol-Paque

(Gibco-Invitrogen). The cells were collected, washed, and seeded in T flasks. The cells were characterized by morphology, adherence, and flowcytometry assessment using CD44, CD90 and CD45 surface markers.

EVs isolation and characterization protocol

EVs were isolated from MSCs supernatant. After filtration, ultracentrifugation was done at $120,000 \times g$ for 70 minutes at 4°C (Thermo Fisher Micro Ultracentrifuge MX 120+, Medical Research Institute- Alexandria University), pellets were discarded, re-suspended in PBS, and stored for -80°C . Transmission Electron Microscope (TEM) with an accelerating voltage of 120 kV (JEM-1400 series 120kV Transmission Electron Microscope, USA) was used for EVs characterization.

miRNA-126 Analysis

The extraction of total RNA from BM-MSCs and their EVs was done under completely sterile and RNase-free conditions in accordance with the manufacturer's instructions of miRNeasy Mini Kit (Qiagen, Hilden, Germany). The evaluation of the concentration of the extracted RNA samples was done using NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA). Quantitative RT-PCR analysis for miRNA-126 was next done twice over using TaqMan microRNA assays and TaqMan Universal PCR Master Mix II (Applied Biosystems, USA) [8]. StepOne real-time PCR system (Applied Biosystems, USA) was used for amplification. StepOne™ Software v2.3 was used for data analysis [9].

BM-MSCs transfection protocol

One day before transfection, BM-MSCs were cultured $2-8 \times 10^4$ cells per well in a 24-well plate. On the day of transfection, 37.5 ng miRNA-inhibitor/mimic was mixed with 2 μL of lipofectamine 2000 transfection reagent (Invitrogen Cat No. 11668-030). After incubation, the complexes were added to the cells and re-incubated for 72 h. qRT-PCR analysis was done as previously described.

The induction of T1DM was done by single intraperitoneal (I.P) injection of streptozotocin (STZ) in a dose of 50 mg/Kg [7]. Healthy untreated rats received an equal volume of phosphate buffer saline (PBS) intraperitoneal and served as controls (group I, control group)(n=10). Diabetic rats were randomly divided into five groups each containing 10 rats. Group II: untreated diabetic group, Group III: Diabetic rats treated with single I.P. injection of BM-MSCs in a dose of 106 cells per rat [10].

Group IV: Diabetic rats received a single I.P. injection of BM-MSCs derived extracellular vesicles at a dose of 1000 μg /ml in a frozen pellet,

Group V: Diabetic rats received single I.P. injection of BM-MSCs derived extracellular vesicles expressing miR-126 mimic with a dose of 1000 μg /ml in a frozen pellet [10].

Group VI: Diabetic rats received single I.P. injection of BM-MSCs derived extracellular vesicles expressing miR-126 inhibitor with a dose of 1000 μg /ml in a frozen pellet.

Animal scarification was done by cervical dislocation at the end

of the study. Blood samples were taken from the aorta in EDTA tubes (10 µl of 0.1 M EDTA for 200-400 µl of blood); plasma was separated for the assessment of insulin level and stored at - 80 °C.

Biochemical analysis:

Plasma insulin level

Plasma insulin level was determined by ELISA according to the manufacturer instructions (Insulin ELISA kit Thermo Fisher, USA). Once rats' plasma insulin level became below 9 µIU/ml, they were considered diabetic.

Blood glucose level (Fasting/ Random)

Blood glucose level was measured weekly using samples from venous blood glucocheck strips.

Histopathological examination of the pancreas

After sacrifice, the pancreatic tissues were harvested, fixed, and processed into paraffin blocks. Four to five microns sections were cut using a manual rotatory microtome. Hematoxylin and eosin (H&E) stained section were then assessed for histopathologic changes [11].

Statistical Analysis

All statistical analysis was done by SPSS software version 20.0. (Armonk, NY: IBM Corp). Mean and standard deviation were used to summarize the data. Analysis of variance (ANOVA) test was done for comparison between two groups with post hoc test (Tukey's) for pairwise comparison. Post Hoc Test (adjusted Bonferroni) for pairwise comparisons was done to compare between two durations. Statistical significance was considered at p values ≤0.05.

Ethical Approval

This study was approved by the Ethics Committee of Alexandria Faculty of Medicine (Date: 2021-6-27, IRB No: 00012098- FWA No: 00018699).

Results

Characterization of Bone marrow mesenchymal stem cells (BM-MSCs)

MSCs were characterized by their fibroblast spindle-shape using inverted contrast phase microscope (Olympus CKX41SF, Japan) (Fig. 1 A-D). Flowcytometric analysis of cultured BM-MSCs showed the positive expression of the mesenchymal surface markers CD44 and CD90 as well as negative expression of CD45 (Fig. 1E-F)

Characterization of Extracellular vesicles

TEM was used to visualize the isolated EVs, showing spherical structures with lipid bilayer of different sizes ranging from 50.92 to 67.03 nm. Showing that most of extracellular vesicles were exosomes (Fig. 1G).

Quantification of miRNA-126 level in BM-MSCs and their EVs miRNA-126 expression in BM-MSCs and their derived EVs as compared to un-transfected BM-MSCs and BM-MSCs transfected with miRNA-126 inhibitor (in Vitro)

Cells transfected with miRNA-126 mimic showed 17-fold higher miRNA-126 expression than BM-MSCs. Additionally, miRNA-126 dropped by 4 folds following transfection with miRNA-126 inhibitor (Fig. 2A).

Evaluation of Therapeutic Effect of BM-MSCs and Their EVs

Plasma insulin level

There was a significant increase in the level of plasma insulin

in BM-MSCs, EVS and

EVs - miRNA-126 mimic treated groups as compared to untreated diabetic group and EVs-miRNA-126 inhibitor group (P ≤ 0.001). (Fig. 2B).

Blood glucose level

There was significant decrease in blood glucose levels in the BM-MSCs, EVs and miRNA-126 mimic treated groups compared to untreated diabetic, and miR-126 inhibitor treated groups (P ≤ 0.001). (Fig. 2C).

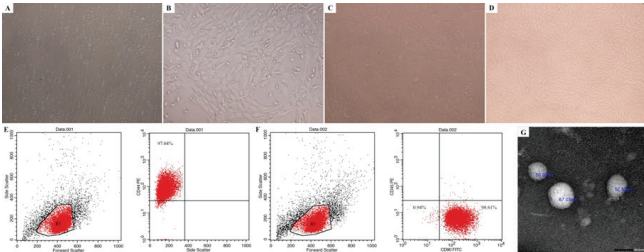


Figure 1. Characterization of BM-MSCs and EVs. Under an inverted phase microscope, the cells showed round and spindle shape morphology (A). After 5 days, the cells became adherent fibroblast-like cells 60 % confluence (B). BM-MSCs increased with 80 % confluence after 7 days' culture (C). BM-MSCs became adherent fibroblast like cells with 100 % confluence after 14 days (D). Representative panel of flow cytometric cell surface markers for BM-MSCs showing the percentage of CD44, CD90 cells, CD45 cells as 97.04%, 98.61%, 0.94% respectively (E-F). EVs visualized using TEM, showing EVs of different sizes ranging from (50.92- 67.03 nm) Scale bar, 50nm (G)

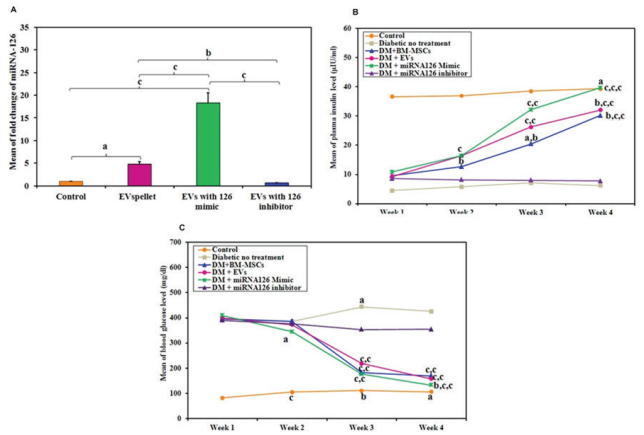


Figure 2. A-C. Evaluation of the therapeutic effect of BM-MSC and their EVs. Comparison between the levels of miRNA-126 expression in cell culture in different groups. Values are mean ± SD. Significant post hoc test is indicated by letters a p ≤0.05, b p ≤ 0.01, c p ≤0.001 (A). Comparison between the different groups according to plasma insulin level. The control group, diabetic group, EVs with inhibitor treated groups showed no significant difference between all durations(p>0.05). BM-MSCs, EVs and EVs with miRNA-126 mimic treated groups showed significant increase in plasma insulin level (p ≤0.001) (B). Comparison between the different groups according to blood glucose level. The control group, diabetic group, EVs with inhibitor treated groups showed no significant difference between all durations(p>0.05). BM-MSCs, EVs and EVs with miRNA-126 mimic treated groups showed a significant decrease in blood glucose level (p ≤0.001) (C)

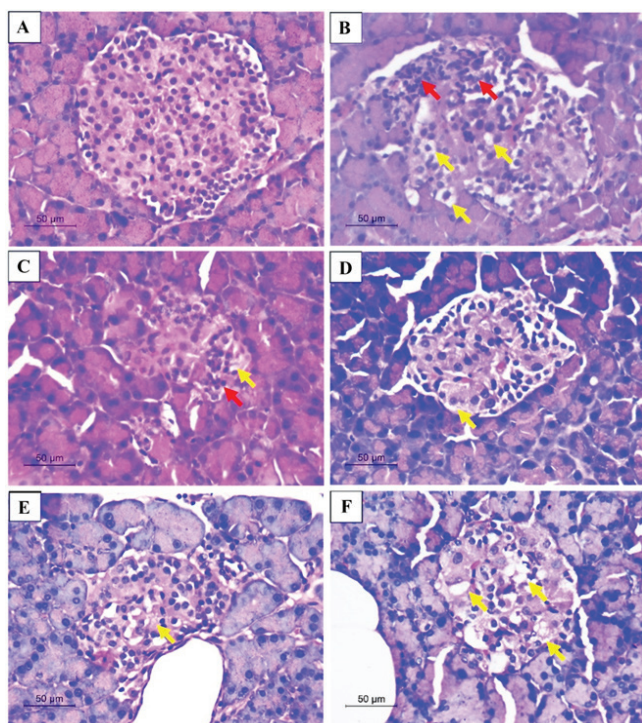


Figure 3. (A-F) Hematoxylin and Eosin(H&E) staining of pancreatic islets (x400). Normal group showing polygonal cells arranged as trabeculae separated by thin vascular spaces. (A), diabetic group showing degenerative changes (B), BM-MSCs treated group showed some improvements in architecture (C). EVs treated group showed improvement of endocrine cell histology where vacuolation is only focally seen (yellow arrow) (D). EVs with mimic showed increased number of islet cells with few cytoplasmic vacuolation (yellow arrow) (E). EVs with inhibitor showed decreased islet cell count and evident vacuolation (yellow arrows) (F)

Histopathological Assessment

Pancreatic tissues of rats treated with miRNA-126 mimic, BM-MSCs and EVs stained with hematoxylin and eosin (H&E) showed improvement compared to untreated diabetic and miRNA-126 inhibitor. Diabetic group showing degenerative changes seen as mononuclear infiltration (red arrow) and diffuse cytoplasmic vacuolation of islets cells (yellow arrow) (x400) (Fig 3B)

BM-MSCs treated group showed improvement in endocrine cell histology where vacuolation is only focally seen (yellow arrow) with residual mono nuclear cells are seen (red arrow) (x400) (Fig 3C). EVs treated group showed improvement in endocrine cell histology where vacuolation is only focally seen (yellow arrow) (Fig 3D). EVs with mimic showed an increased number of islet cells.

Discussion

T1DM treatment is considered a challenge mainly due to the early onset of the disease and its unknown exact etiology. Many different treatment modalities were used for the treatment of T1DM [1]. BM-MSCs have been recognized as a hopeful agent for the treatment of T1DM because of their ability to differentiate into islets-like cells. MSCs regenerative ability can be attained through certain paracrine factors released from them, which include extracellular vesicles (EVs). EVs derived from MSCs have immunomodulatory effects through induction of T-regulatory

cells and suppressing autoreactive immune cells [12]. MiRNAs may have shown an important role in many aspects of diabetes as they modulate the expression of many genes associated with the pathophysiological process. In the current work, EVs were used as cargo for miRNA-126 mimic and inhibitor to prevent their digestion by different nucleases and avoid their modification [13].

In the current work, T1DM was induced by STZ injection. Many previous studies have used STZ for induction of T1DM because of its cytotoxic effect in β -cells through inflammation activation, production of excessive amount of reactive oxygen species, and cell apoptosis [14, 15].

In the present study, T1DM was confirmed by high blood glucose >300 mg/dl and decreased plasma insulin level and histopathological pancreatic changes. These results were consistent with previous studies that reported the confirmation of T1DM was done by fasting blood glucose level >300 mg/dl and histopathological pancreatic changes [15-16-17].

The results of the present work revealed that cultured BM-MSCs were characterized by their morphology and surface markers (Fig. 1 A-F) which was consistent with a previous study by Kobolak et al. [18]. EVs were also characterized by their morphology (Fig 1G) which agreed with previous study that denotes the size of EVs derived from BM-MSCs were ranged 100 nm in diameter with typical spherical shape [7].

The study results documented that BM-MSCs treated group showed significant lowering in blood glucose levels and an elevation in plasma insulin levels, as well as improvement in histopathological pancreatic tissue findings. This may be due to their immunomodulatory effects, their homing capabilities [4, 12]. These findings are in agreement with previous studies that revealed the decline in blood glucose level and elevation in plasma insulin level after injection BM-MSCs in T1DM rats [7, 19].

Regarding the other group that received EVs, they showed decrease in blood glucose level, increase in plasma insulin level and improvement in histological findings in pancreatic tissue. However, EVs results were superior to those of BM-MSCs results which may be due to the immunomodulatory ability of EVs through inhibition of reactive T cells and reduction of inflammatory cytokines levels [20].

Rani et al. [21] stated that exosomes can be used as a novel substitute to whole cell therapies. Exosomes may provide a better safety profile when compared to their parent cells. The previously mentioned study could provide explanation for the mechanism of improvement that occurred in our present work in EVs treated group [21].

The findings of the present study demonstrated that miRNA-126 mimic treatment has improved fasting blood glucose, plasma insulin levels and pancreatic tissue histopathology, which denoted possible therapeutic effects. Other previous study had reported the anti-inflammatory role of miRNA-126 as denoted by intravitreal injection of miRNA-126 mimic has improved diabetic retinopathy by enhancing retinal tissue histopathology and inflammation by inhibiting specific inflammatory pathways [22].

Another previous study suggested that the increase in expression of miRNA-126 may play anti-inflammatory role,

antiapoptotic role in cardiomyopathy thus this could explain the mechanism of miRNA-126 in reversing cytotoxic effects of STZ on islets cells [23].

Zhang and his colleagues [24] have revealed the angiogenic role of miRNA-126 as they reported that diabetic wounds treated with negative pressure wound therapy (NPWT) revealed large increases in miR-126, which controls VEGF-induced angiogenesis [24]. Thus these previously mentioned findings may be provide a possible explanation to the results in our present study in terms of improvement of β -islet cell mass after treatment of miRNA-126 through angiogenesis.

Conclusion

The current study highlights miRNA-126 mimic- transfected EVs as a promising, safe, cell-free treatment strategy for T1DM. In addition, molecular action of EVs could be mediated by miRNA-126 in T1DM rat model. However, the exact molecular pathways and mechanisms of miRNA-126 should be further investigated.

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Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and Human Rights Statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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